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Characterization of Circulating *Clostridium difficile* Strains, Host Response and Intestinal Microbiome in Hospitalized Children With Diarrhea

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Background: *Clostridium difficile* is capable of causing severe enterocolitis in adults. The significance of toxin-producing *C. difficile* in children with diarrhea is unclear and practice differs on whether to institute treatment. We aimed to characterize the microbiome in relation to the presence of *C. difficile* and co-infection with other pathogens and to describe host response to infection.

Methods: Participants were children with acute diarrhea, 0–16 years of age, from whom stool samples had been submitted to the hospital laboratory for routine microbiology/virology. Convenience sampling was used for 50 prospective and 150 retrospective samples. No participants were treated for *C. difficile*. Rates of culture positivity for *C. difficile*, presence of toxin and PCR-ribotype were compared between age groups. Presence of other potential pathogens, comorbidities and complications were recorded. Microbiotal diversity was measured by 16S profiling.

Results: Nineteen of 77 (25%) children <2 years of age and 13 of 119 (11%) children >2 years of age were *C. difficile* positive, of whom 10 (53%) and 9 (69%), respectively, carried toxigenic strains. Increased Shannon diversity was seen in children carrying *C. difficile,* with altered milieu. Presence of *C. difficile* was not associated with adverse clinical outcomes. In stools containing both Norovirus and *C. difficile*, there was increased relative abundance of verrucomicrobia.

Conclusions: Children with diarrhea regularly carried toxigenic and nontoxigenic strains of *C. difficil*e, demonstrating enhanced microbiotal diversity, and change in milieu, without apparent morbidity. This unexpected finding is contrary to that seen in adults with *C. difficil*e disease.

Key Words: *C. difficile*, diarrhea, children, host response

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Clostridium difficile (*C. difficile*); a Gram-positive, anaerobic, spore-forming bacillus, existing in both toxigenic and nontoxigenic forms, is a significant cause of nosocomial infection and associated morbidity/mortality; particularly in the elderly following the emergence of hypervirulent strains such as PCR ribotypes 078 and NAP1/B1/027.[1,2] Healthy neonates and infants frequently carry *C. difficile* (including toxin-producing strains), as part of their bowel flora, without apparent adverse effects. The mechanisms by which this occurs remain obscure, with immaturity of receptors for *C. difficile* toxin in the infantile gut[3] or a protective effect of breast milk via transfer of neutralizing antibodies to toxins A and B postulated as major contributors.[4,5] *C. difficile* infection (CDI) requires alteration of the colonic flora, colonization by toxigenic *C. difficile* and release of enterotoxins (Toxin A and/or Toxin B) for disease establishment. Broad-spectrum antibiotic therapy eliminates intestinal commensals, allowing overgrowth of *C. difficile* and potential CDI. It has been reported that relapse/recurrence of CDI in adults occurs in those with low levels of IgG/IgM to *C. difficile* toxins.[6] Seropositivity rates increase with age, with 11% and 33% of infants <6 months of age displaying antibodies to Toxin A and B, respectively, increasing to 25% and >50% seropositivity by 2 years of age.[7]

Asymptomatic *C. difficile* carriage decreases with increasing age; up to 70% carriage has been reported in healthy neonates,[3] falling to around 10% at 1 year, and reaching comparable rates with adult colonization of 4% by 2 years. Despite a global decrease in CDI rates, incidence appears to be increasing amongst traditionally "low risk" groups for infection, such as children and adults in the community.[8] There have also been more cases in hospitalized children with known "risk factors" for infection, such as hematologic malignancies, inflammatory bowel disease and antimicrobial use.[9] Rising reported rates of CDI[10] may be partially due to the introduction of more sensitive nucleic acid amplification-based testing. *C. difficile* is currently on the Center for Disease Control's list of "urgent threats" to US public health.[11]

The gut microbiome is frequently less diverse in adult patients with CDI.[12–14] The pediatric gut microbiome is influenced by numerous factors, including gestation, mode of delivery at birth, method of feeding, medications and environmental factors, but whether the presence of *C. difficile* also influences this remains unclear. Broad groups of bacteria are largely similar across

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individuals, but there is a great degree of diversity and individuality in the acquisition of species over time.[15]

C. difficile is often treated as a pathogen in children with diarrhea, particularly in the United States,[16] but this may be unnecessary. In hospitalized children with diarrhea and *C. difficile* in their stool, we aimed to characterize the microbiome in relation to co-infection with other pathogens and to describe host response to infection.

PATIENTS AND METHODS

Setting

Alder Hey Children's NHS Foundation Trust Hospital is a university-affiliated pediatric teaching hospital in the north-west of England, undertaking >275,000 patient care episodes per annum.[17] We recruited children 0–16 years of age experiencing acute diarrhea, with samples submitted for microbiologic testing at the request of medical staff.

Prospective Study

Fifty stool samples sent for stool microbiology/virology testing were collected between November 2013 and January 2014 from inpatient diarrheal specimens. Samples with sufficient yield following routine testing were immediately stored at −70°C. If the child had undergone blood tests that day, any remaining plasma was stored at −20°C. Samples were anonymized after extraction of clinical data and analyzed at a later date.

Retrospective Study

One hundred and fifty stool samples from children with diarrhea submitted for stool microbiology/virology between January and July 2013 were collected. Samples were from hospitalized children and Emergency Department attendees. Four samples were found to be duplicates and removed from the study (ie, same patients on the same admission). *C. difficile* testing is not routinely done on these samples. None of the children were treated for CDI (see Figure, Supplemental Digital Content 1, http://links.lww.com/INF/D729, for sample flow).

Patient demographics and data on comorbidities and medication use were obtained for both groups.

Ethical Statement

Study design was reviewed and approved by the NHS Research Ethics Committee London—Fulham (reference: 13/LO/1495) and the Research and Development departments of Alder Hey Children's NHS Foundation Trust and the University of Liverpool. Written informed consent was taken from parents of all participants in the prospective study and written assent from participants, where age-appropriate.

Sample Analysis

Stool Culture and *C. difficile* Detection

All stool samples underwent alcohol-shock treatment, followed by culture for *C. difficile* on Brazier's cefoxitin-cycloserine egg yolk agar as previously described.[18] Samples underwent lateral flow testing for the presence of *C. difficile* glutamate dehydrogenase and the presence of toxins A and/or B with C. diff Quik Chek Complete (Alere LTD, Stockport, United Kingdom), following manufacturers' instructions.

Fecal Cytokines

Stool supernatants from 72 samples; 30 *C. difficile*-positive (with sufficient remaining sample) and 42 *C. difficile*-negative samples (aged within 1 year of positives) underwent testing for fecal cytokine levels with the V-PLEX Pro-inflammatory panel 1 (Meso Scale Discovery, Rockville, MD). Briefly, 50 µl/0.05g stool were suspended in 250 µl PBS/Sigmafast protease inhibitor (Sigma-Aldrich Ltd, Dorset, United Kingdom), and following vortexing and centrifugation, the resulting supernatant was utilized in the kit as per manufacturers' instructions.

Molecular Methods

PCR assays for toxin genes, ribotyping, 16S parallel sequencing and testing for additional pathogens using the Luminex Gastrointestinal pathogen panel (GPP) were completed on 59 samples. Methods and sample selection procedure are described in detail in online Appendix 1.

Statistical Analysis

All results were analyzed using STATA (release 14·0, STATA Corp., College Station, TX) or GraphPad Prism (release 7·0a, GraphPad Software Inc., La Jolla, CA). Data which were parametrically distributed were examined with *t* test, while Kruskal-Wallace/Dunn test was employed for non-parametrically distributed data, with a P value <0.05 considered significant. See Supplemental Digital Content 2, http://links.lww.com/INF/D730 for statistical methods for 16S data.

RESULTS

Demographics and Patient Characteristics

Participants ranged from 0 to 16 years of age (Median 3·0; IQR 0.83–9); 77 (40%) were <2 years of age, and 119 (60%) ≥2 years of age. Of the 32 samples testing positive for *C. difficile,* 21 (64%) were sent within 72 hours of admission (see Tables 1 and 2 for further patient demographics by group). *C. difficile* positivity was not associated with increased length of stay.

TABLE 1. Population Demographics by Group (<2 Years)

	Culture negative (CN) (n = 58)	Non-toxigenic carriers (NTC) (n = 9)	Toxigenic carriers (TC) (n = 10)	*P* (Dunn test)
Age (months)	5.52 (IQR 2.04–12.48)	8.99 (IQR 8.52–15.96)	12 (IQR 7.80–14.28)	0.03 (CN vs. NTC), 0.008 (CN vs. NTC/TC)
Sex (male)	29/58 (50%)	7/9 (78%)	4/10 (40%)	NS
Length of stay (days)	8.5 (IQR 1.75–43)	2 (IQR 0–6.5)	6.5 (IQR 2–25.75)	0.03 (CN vs. NTC)
Time to sample (days)	3 (IQR 1–18.5)	1 (IQR 0–2)	2 (IQR 0–8.5)	0.01 (CN vs. NTC)
Comorbidities*	38/75 (51%)	5/9 (56%)	6/10 (60%)	NS
Current antibiotics	14/68 (21%)	4/7 (57%)	4/8 (50%)	0.04 (CN vs. NTC), 0.07 (CN vs. TC)
Antibiotics past 3 months	34/58 (59%)	6/6 (100%)	4/5 (80%)	0.05 (CN vs. NTC)
Current PPI/H2RA	18/68 (26%)	2/7 (29%)	3/8 (38%)	NS
Currently PEG/JEJ fed	16/71 (23%)	3/9 (33%)	3/9 (33%)	NS

*Comorbidities considered were diagnoses in the following categories: hematologic, neurologic, gastrointestinal, respiratory, cardiologic and other.

TABLE 2. Population Demographics by Group (>2 Years)

	Culture negative (CN) (n = 106)	Non-toxigenic carriers (NTC) (n = 4)	Toxigenic carriers (TC) (n = 9)	*P* (Dunn test)
Age (years)	8 (IQR 4.13–12)	8 (IQR 7–11.25)	4.42 (IQR 3.08–7.5)	NS
Sex (male)	58/106 (55%)	4/4 (100%)	1/9 (11%)	0.08 (CN vs. NTC) 0.01 (CN vs. TC), 0.003 (NTC vs. TC)
Length of stay (days)	7 (IQR 0–20)	12 (IQR 3–46.5)	8 (IQR 4.5–129)	NS
Time to sample (days)	1 (IQR 0–8.25)	4.5 (IQR 1.75–8.75)	3 (IQR 1.5–52.5)	0.04 (CN vs. NTC)
Comorbidities	67/106 (63%)	4/4 (100%)	9/9 (100%)	0.03 (CN vs. TC)
Current antibiotics	37/99 (37%)	2/4 (50%)	4/9 (44%)	NS
Antibiotics past 3 months	59/60 (98%)	4/4 (100%)	7/8 (88%)	NS
Current PPI/H2RA	32/99 (32%)	4/4 (100%)	6/9 (67%)	0.005 (CN vs. NTC), 0.04 (CN vs. TC)
Current PEG/JEJ fed	26/104 (25%)	4/4 (100%)	5/9 (56%)	0.001 (CN vs. NTC), 0.05 (CN vs. TC)

In the <2-year group, 19 (25%) samples tested positive for *C. difficile* (defined as positive result on both culture and lateral flow testing), of which 10 (53%) were toxigenic strains. In the >2-year group, 13 (11%) samples tested positive for *C. difficile,* of which 9 (69%) were toxigenic strains. PCR-ribotypes were more diverse in the >2-year olds, in whom 12 different strains were identified with only type 002 being detected twice. In contrast, 039 (9 samples) and 020 (6 samples) were identified most frequently in the <2-year olds (Fig. 1A,B).

There was a significant difference in median age of those <2 years testing positive *for C. difficile;* 0.83 (IQR 0.67–1.25) versus 0.42 (IQR 0.17–1.04) years for those testing negative ($P = 0.008$). Male patients were significantly more likely to carry non-toxigenic *C. difficile*; 11 of 13 (85%) carriers were male versus 87 of 164 (53%) culture negative ($P = 0.01$).

There was an association between comorbidity and carriage of toxigenic *C. difficile* in those >2 years; 63% (67/106) culture-negative patients had comorbidities versus 100% (9/9) patients with a toxigenic strain ($P = 0.03$). All children over 2 years with *C. difficile* had a comorbidity, these included malignancy, cerebral palsy or other congenital syndromes causing multisystem disorders. A significant proportion of culture-negative children >2 years (63%) also had a comorbidity; however, with a similar spread of conditions, as well as some children with gastrointestinal disorders.

Maximum C-reactive protein (CRP) was significantly higher in children carrying toxigenic *C. difficile*; CRP 10.1 (IQR 4–61.8) in culture negative, 9.5 (IQR 4–52.2) in non-toxigenic and 34.8 mg/L (IQR 4–144.4) in toxigenic carriers ($P = 0.04$) (Fig. 2). There was a non-significant association between toxigenic carriage and higher white cell count (WCC).

Recorded diagnoses for all participants were as follows: Unspecified loose stools 55 of 196 (28%), gastroenteritis (no reported organism) 19 of 196 (10%), gastroenteritis (organism identified) 12 of 196 (6%), upper/lower respiratory tract infection 33 of 196 (17%), sepsis 13 of 196 (7%) and other 64 of 196 (33%).

Coinfections

Using the GPP panel, pathogens were identified in 27 of 59 (46%) of the 59 stools tested, with norovirus being detected in 21 of 59 (36%). *C. difficile*/norovirus co-infection occurred in 9 of 21 (43%) of these cases (see Table, Supplemental Digital Content 3, http://links.lww.com/INF/D731).

Co-infection with virus, but not bacteria was associated with presence of *C. difficile*, ($P = 0.001$), and also the presence of a toxigenic strain ($P = 0.004$). There was no association between elevated WCC or CRP and presence of co-infection.

Fecal Host Response Biomarkers

Children with a diagnosis of inflammatory bowel disease had higher levels of both pro- and anti-inflammatory cytokines (TNFα, IL-4, IL-6, IL-8, IL-12, IL-1β, IL-13, IL-12p70, IL-10 and IFNγ, all $P < 0.0025$). There was no clear pro-inflammatory response in those with *C. difficile,* versus those without, regardless of toxin status, with no significant difference noted in cytokine levels between groups.

16S Sequencing Results

Taxonomic data were available for 42 children; these were split into 3 groups for analysis: 18 culture negative, 9 carrying non-toxigenic *C. difficile* and 15 carrying toxigenic *C. difficile*. Data were analyzed in terms of Shannon diversity index, which incorporates both richness of diversity (ie, number of species present) and their relative abundance. Chao1 and Species Abundance Coverage Estimator were also reported on, which are alternative methods of estimating true species diversity and abundance, respectively.

Shannon diversity index was higher among children who were culture positive [toxigenic or non-toxigenic ($P < 0.05$)], but Chao1 and Species Abundance Coverage Estimator species richness indices did not differ significantly across the 3 groups (Fig. 3).

At the phylum taxonomic level, culture negative and non-toxigenic carriers of *C. difficile* demonstrated increased proportions of Firmicutes and Bacteroidetes, with fewer Proteobacteria. In toxigenic *C. difficile* carriers, the converse was true, with a marked increase in abundance of Proteobacteria. In *C. difficile* and norovirus co-infection, there was a bloom in Verrucomicrobia; this was seen to a lesser degree in those with norovirus alone, but not witnessed in any other group. Those who were norovirus positive alone also displayed an increase in abundance of Proteobacteria with a concomitant decrease in Actinobacteria. Conversely, Actinobacteria appeared to be more abundant in *C. difficile*-positive patients (Fig. 4; Figure, Supplemental Digital Content 4, http://links.lww.com/INF/D732).

Additionally, Lachnospiraceae and Ruminococcaceae family abundance was significantly higher in those who were carrying either toxigenic or non-toxigenic *C. difficile* ($P < 0.001$ and $P < 0.006$, respectively). The genus *Klebsiella* was significantly more abundant in carriers of toxigenic strains of *C. difficile* ($P < 0.006$).

The coverage depth of our sequencing data allowed the confirmation of *C. difficile* presence at very low levels (maximum relative abundance of 0.003%), and detected 2 further carriers who were negative on culture/lateral flow testing.

DISCUSSION

Key findings of this study were increased Shannon diversity index in *C. difficile*-positive children with diarrhea (particularly

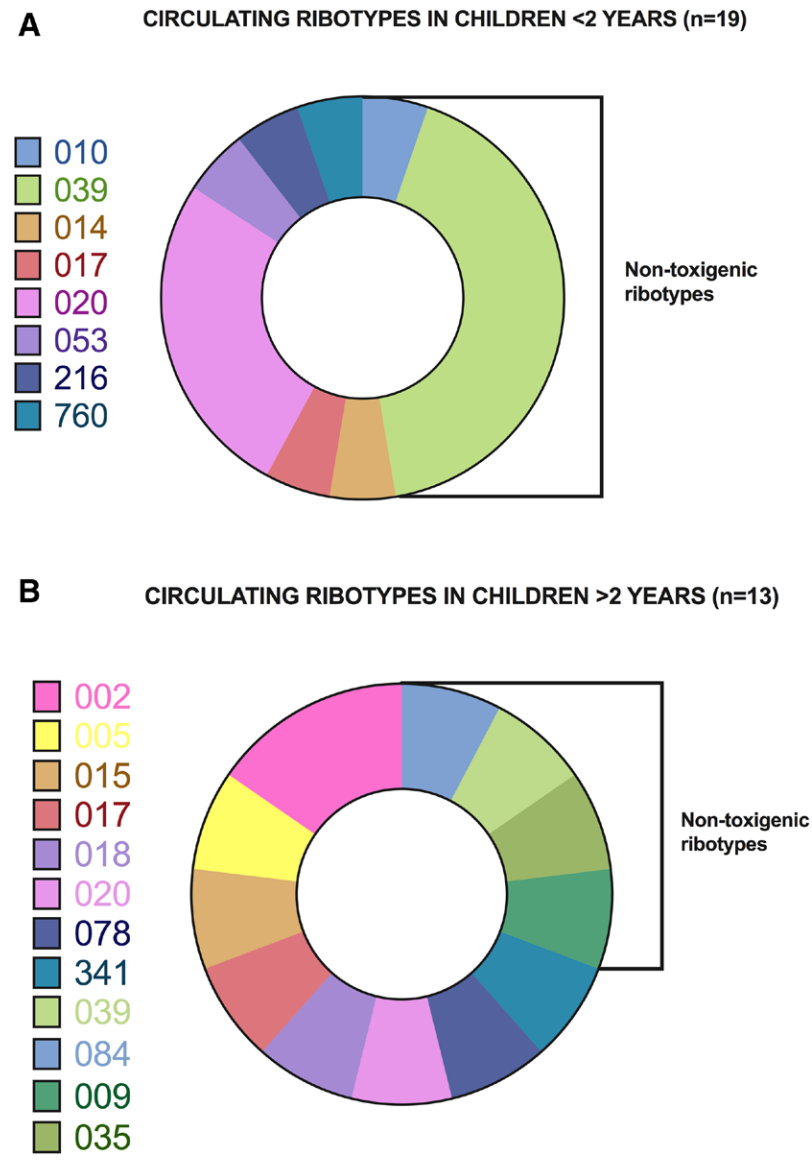

FIGURE 1. Figures representing diversity of *C. difficile* strain isolated, investigation of systemic response and effect of *C. difficile* on microbiota. A: PCR-ribotypes identified in children <2 years old. B: PCR-ribotypes identified in children >2 years old. PCR-ribotypes identified in children >2 years old were much more diverse than those detected in children <2 years old, suggesting sharing of strains between children <2 years old before toileting/hand hygiene habits are learnt. full color online

non-toxigenic carriers), with change in milieu, and a Verrucomicrobial bloom in those co-infected with *C. difficile*/norovirus.

A study of gut microbiota in 53 healthy infants showed no decrease in global diversity with the presence of *C. difficile*, and similarly to our study, an increased abundance of *Klebsiella* and *Ruminococci*[19] Findings in children contrast with those in adult *C. difficile* patients, in whom the diversity of the microbiota is diminished, with decreased abundance of Ruminococci reported.[20,21] A high relative abundance of Lachnospiraceae has been suggested to be protective against *C. difficile*.[22,23] This may reflect the fact that in adults, the cause of diarrheal disease was *C. difficile*, whereas this was not thought to be the case in our pediatric patients. None of the children in our study were treated for CDI. There is no clear reason why *C. difficile* does not appear to affect the diversity of the pediatric gut microbiome in the way that it does in adults; one explanation could be that the bacteria is preferentially existing in spore form in the pediatric gut, with conditions not being optimal for proliferation of the vegetative form.[24] One longitudinal study of the microbiota of an asymptomatic infant covering periods of breast-feeding and weaning found carriage of 2 different strains of *C. difficile* over the study period at both high vegetative and spore counts, with presence of toxin levels that would be high enough to cause disease in adults. In this case, change in microbiotal composition associated with alteration of diet from breast milk to cow milk caused rapid expulsion of *C. difficile* from the bowel.[25]

Temporal and spatial overlap between norovirus outbreaks and CDI, which may be potentially synergistic, has been documented previously.[26] Norovirus and *C. difficile* co-infection have been reported in other pediatric studies,[27,28] but those with co-infections were clinically indistinguishable from those with *C. difficile* alone, despite the higher bacterial burden in co-infection. This suggests that Norovirus leads to a loss of colonization resistance to *C. difficile*, rather than a dysbiosis. Also noted during our study was that proportions of *Firmicutes* and *Bacteroidetes* decrease and *Proteobacteria* increase in children with toxin-positive *C. difficile*. This raises the possibility that these differential abundances in the presence of toxin could drive disease, as opposed to the presence of *C. difficile* per se.

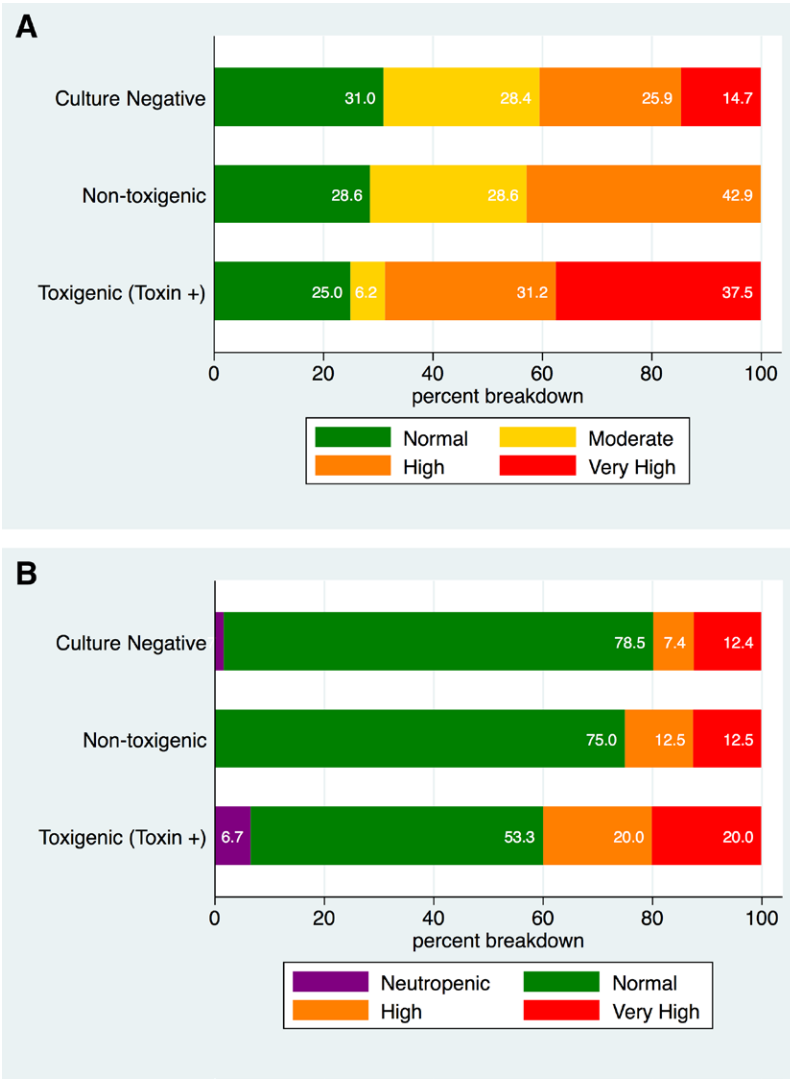

FIGURE 2. Figures representing diversity of *C. difficile* strain isolated, investigation of systemic response and effect of *C. difficile* on microbiota. A: CRP by *C. difficile* status. Maximum CRP was significantly higher in children carrying toxigenic strains of *C. difficile* versus those with non-toxigenic strains and those who were culture negative ($P = 0.04$). Note: CRP defined as normal (<4), moderate (4–20), high (20–100) and very high (>100). B: WCC by *C. difficile* status. Those with toxigenic *C. difficile* had a higher WCC in but this was not significant. The increased prevalence of neutropenia in this group likely represents the fact that hematologic comorbidity was associated with toxigenic *C. difficile* carriage. Note: WCC defined as neutropenic ($<1.5 \times 10^9$), normal ($1.5–15 \times 10^9$), high ($15–20 \times 10^9$) and very high ($>20 \times 10^9$). full color online

Our data suggest *C. difficile* may be a bystander in diarrhea caused by other primary pathogens; supported by the finding of similar carrier frequency in the "healthy" outpatient population in a number of studies.[29,30]

The proportion of children who tested positive for *C. difficile* within 72 hours of admission fits with the current increase in cases of community-associated *C. difficile* being identified in adults and children in the United States.[1,8,16] Prevalence of *C. difficile* in the age-stratified population studied here fit with those found in an extensive literature review.[29]

We only found an association between current antibiotic use and *C. difficile* carriage in the <2-year age group in this study; largely because of the very high prevalence of recent antibiotic use in both groups. More detailed information on class of antibiotic used would have been valuable to correlate with *C. difficile* status. Another factor of interest would be repeated sampling over time around a course of antibiotics to observe changes in microbiota associated with treatment.

A limitation of our study is that not all samples underwent GPP panel testing or 16S sequencing. Given the retrospective nature of many of the samples, we were unable to determine whether all participants had been in contact with hospitals in the past 3 months, so the proportion with community-associated carriage may be overestimated.

The significantly higher CRP in those found to carry toxigenic *C. difficile* raises the question of whether CRP is elevated in relation to intestinal inflammation due to presence of *C. difficile* toxin, or whether systemic inflammation provides favorable conditions for *C. difficile* colonization. A study on children with diarrhea following antibiotic use noted a significantly higher CRP in the group in whom toxigenic *C. difficile* was detected, with similar demographics between groups, although no data on outcome were available.[31]

It has been suggested that children could form a reservoir for infection in adults, with a higher prevalence of community-acquired CDI in those adults who had contact with children <2 years of age.[32] A large UK study of CDI cases demonstrated that in those with fewer than 2 single nucleotide variants between infecting strains (consistent with transmission), 36% patients had no hospital/community contact with one another, suggesting an alternative reservoir in the community which could be pediatric.[33] A follow-up study found 50% of *C. difficile* strains from healthy infants shared a common lineage with a local CDI case within the past 5 years.[34] We compared our data to unpublished data gathered from hospitalized adults in the same region and found a large degree of overlap

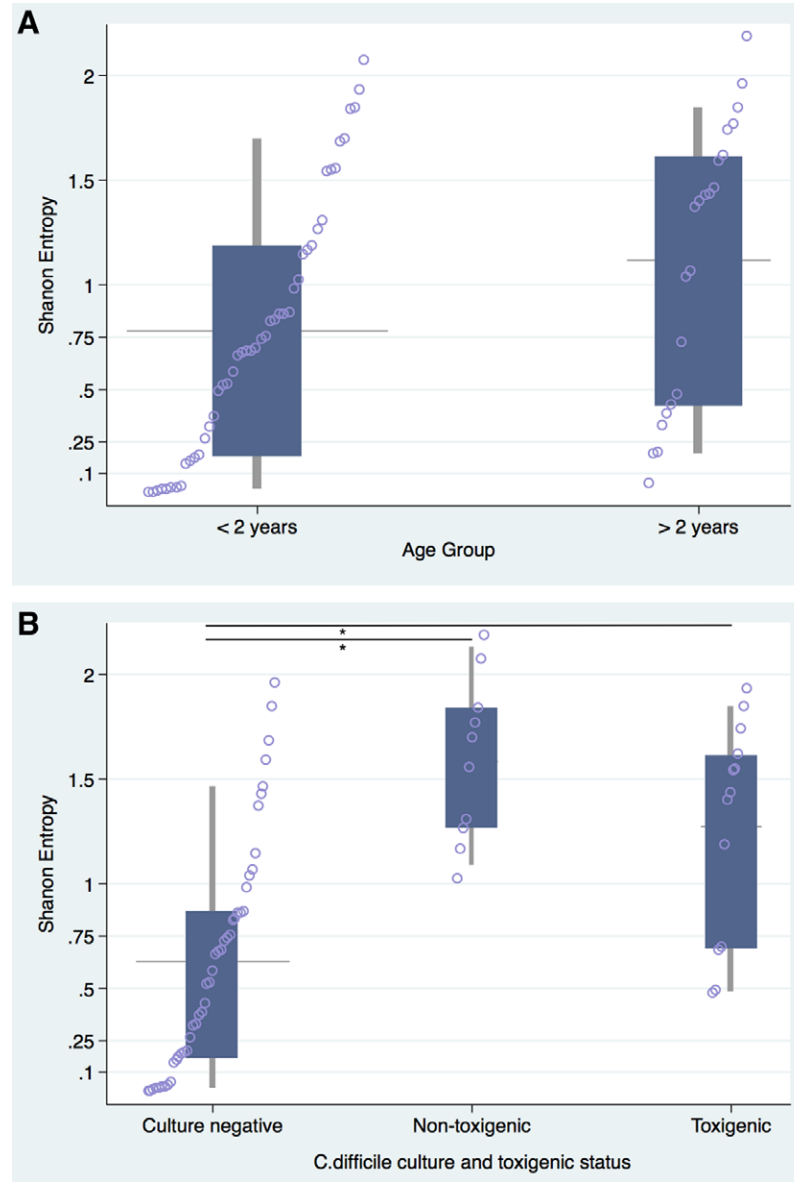

FIGURE 3. Figures representing diversity of *C. difficile* strain isolated, investigation of systemic response and effect of *C. difficile* on microbiota. A: Shannon entropy by age. B: Shannon entropy by *C. difficile* status. Strip plot of Shannon entropy between groups, circles represent individual data points, box 25th–75th centile and whiskers 5th–95th centile. There was no difference in Shannon entropy between age groups, but entropy was significantly higher in those carrying *C. difficile,* regardless of toxin status. Note: *$P < 0.05$. full color online

in strains detected between these adults and children in our study, suggesting transmission of strains between children and adults in our region. In accordance with our findings in the <2-year group, ribotype 020 was also one of the most prevalent ribotypes found in large scale European and Korean studies of hospitalized adults and a Spanish study of children under 2 years.[35–37] In a US study of hospitalized and community adult stool samples, 020 and 039 were found to be the third and fourth most prevalent ribotypes, respectively.[38]

C. difficile PCR-ribotypes found in children >2 years of age in this study were much more diverse than in those <2 years, suggesting that there is less sharing of strains once toileting and hand hygiene habits are learnt.

Infants and children with diarrhea regularly carry toxigenic strains of *C. difficile* without apparent adverse effects. None of the children in this study were diagnosed with or treated for CDI and although higher CRP was associated with toxigenic carriage, there was no increase in local inflammatory cytokine response, which has previously been used to predict disease severity.[39] Interestingly, a recent pediatric study found specific but not sensitive elevation of phosphorylated-p38 in both healthy and diarrheal children with *C. difficile.* The only measure correlating with prolonged diarrhea in those with *C. difficile* was abundance of CXCL-5 mRNA. Bacterial burden did not differ between healthy and symptomatic children and did not correlate with clinical outcome.[40] In our study, carriage conferred no morbidity in terms of length of hospital stay. Other studies have shown no difference in outcome in diarrheal children <2 years of age with toxigenic *C. difficile* in their stool who were treated, versus those left untreated.[37]

Further studies comparing age-matched healthy controls with or without *C. difficile* carriage versus those with diarrhea would provide further insight as to the significance of *C. difficile* in the pediatric bowel, and may contribute to the development of guidance on which children require treatment. These results would be of particular relevance to practice in the United States where pediatric CDI is diagnosed more frequently and treated more aggressively.

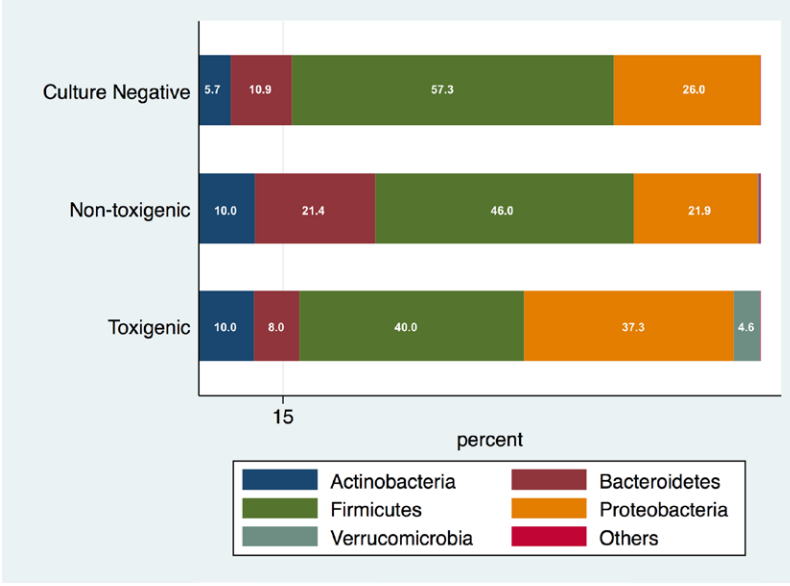

FIGURE 4. Taxonomic distribution by *C. difficile* status. Taxonomic distribution showing relative abundance by phylum versus *C. difficile* status, demonstrating increased abundance of Proteobacteria and decreased Firmicutes/Bacteroidetes in those carrying toxin positive *C. difficile*. Additionally, there is increased abundance of Actinobacteria observed in those who are *C. difficile* positive, which is abrogated in those co-infected with Norovirus (see Figure, Supplemental Digital Content 4, http://links.lww.com/INF/D732). full color online

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